**Lab: Protein purification**

**Objective**

The purpose of this lab is to purify the protein that you overexpressed in bacteria last week. In this lab you will: break open the bacterial cells to release the soluble proteins; remove the insoluble cell debris (cell wall, membranes, inclusion bodies) by centrifugation; and purify the protein using the affinity tag and Ni-NTA resin. During the course of the lab you will collect samples from the different purification steps to analyze in next week’s lab.

**Lab Safety**

Wear a lab coat and gloves while handling bacteria and wash your hands after removing your gloves and before exiting the lab. The bacterial waste must be treated and disposed of properly: liquids should be treated with a 10% bleach solution for 20 minutes; solid waste should be collected in the orange waste bag for autoclaving.

**Background**

The bacteria that you grew last week overexpressed a purple protein (called gbr22) originally isolated from a coral on the Great Barrier Reef. This protein has been modified to have six histidine residues added to the C-terminus, which can be easily used to separate the protein from other cellular proteins. The histidine residues will bind to divalent cations such as nickel that can be immobilized on a column matrix such as nitriloacetate agarose (commonly referred to as Ni-NTA agarose). The protein can be released from the Ni-NTA agarose by adding imidazole, which essentially competes with the histidine residues for metal binding. The affinity of the 6xHis-tagged protein for divalent nickel ions is the basis for the purification protocol that you will use.

In this week’s lab you will break open (lyse) the E.coli cells that express the purple protein, clarify the lysate by centrifugation, purify the protein using Ni-NTA affinity chromatography, and store the purified protein at 4°C. You will also collect samples from the different purification steps to analyze in next week’s lab wherein you characterize the protein using gel electrophoresis and by UV-Vis spectroscopy.

You have the option of working alone or in pairs. However, if you work in a pair – be sure to save your **OWN** samples for each step. You will need them for the 3rd Protein Lab.

**Lab Timeline:**

20 minutes ------- 15 minutes -------- 20 min -------------1.5 hrs

Incubation --------Incubation -----------Spin --------------Purification

Absorbance measurements: **45 minutes**

(can be done immediately after or on another day – but if Nanodrop® is free, you should go ahead and complete this step.)

**Prelab calculations**

1. Calculate the amount of 1 M imidazole and 10x PBS and water to use to make 10 ml of a solution that is 1x PBS and 20 mM imidazole.
2. Calculate the amount of 1 M imidazole and 10x PBS and water to use to make 10 ml of a solution that is 1x PBS and 250 mM imidazole.
3. Determine the second wavelength you will need to use in the lab by completing the MAXIMAL WAVELENGTH section at the end of the lab.

**Materials**

Ice bucket, Beaker of RT (Room Temp) water, 1M Imidazole, 10x PBS, 1x PBS,

**NOTE:** Label all tubes well. We will use several and it can be confusing.

1.7 ml centrifuge tubes

1 x **14 ml** round bottom tube – transformation tube (sterile, expensive)

2 x **10 ml** round bottom tubes – (non-sterile, cheap) – label with tape since you will reuse them

4 x 15 ml conical tubes – to save samples and make stocks – (purple caps)

Bio-rad Econo chromatography column with yellow cap and clear round top

Ring stand and clamps

Ni-NTA resin

**Cyanase** (or Benzonase) – **keep on ice (don’t leave out for more than 10 minutes**

**Q:** Where is your protein now?

**Experimental procedure**

Lysing the *E.coli* cells: Thaw one of the 50 ml conicals containing the 2.5 ml suspension of cells that you prepared last week and have stored in the -20°C freezer by placing in a beaker of water. (Save the other one as backup). The lysozyme that you added will digest the cell walls.

**NOTE**: if you didn’t add lysozyme in the last lab – just add it now.

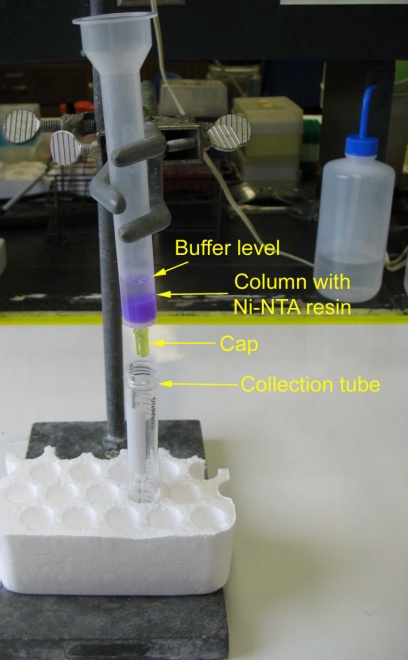
Incubate the solution for **20 minutes** at room temperature mixing occasionally to ensure complete lysis. The mixture should turn viscous when the cells are lysed due to high molecular weight DNA being released from the cells. **Cyanase** (or Benzonase) is a nuclease that will reduce the viscosity by digesting the DNA/RNA in the mixture. Add 50 units (2 μl) of **Cyanase** (or Benzonase) to the 50 ml conical, mix well by inverting the tube several times and incubate for an additional **15 minutes** at room temperature. **Cyanase** (or Benzonase) needs to remain cold as it is an enzyme. So, keep it on ice when dispensing and return it to the -20oC freezer when done. If cell lysate is still ‘sticky’ add 50 units more of **Cyanase** (or Benzonase) and incubate longer.

Return **Cyanase** (or Benzonase) to freezer when done with it.

Clarifying the lysate: Distribute the lysate from the above procedure into several 1.7 ml microcentrifuge tubes and centrifuge for 20 minutes at 14,000 rpm at 4°C in the small centrifuge. After centrifugation, take a 50 μl sample of the supernatant, dispense into a microcentrifuge tube and label it as **sample 2** along with the date and your initials. Store this sample in the 4oC fridge with the one from last week – you will use it in the 3rd protein lab.

Isolating the Soluble Fraction: Using a P1000, transfer the liquid supernatant from the tubes into a clean 15 ml conical tube leaving the cell debris pellets behind. Try not to disturb the pellets; it is better to leave some of the liquid behind than to contaminate the soluble protein with insoluble material. The microcentrifuge tubes containing the cell debris should be disposed of in the red EHS bags.

**Q:** Where is your protein now?



Preparing buffers While you are waiting for the above steps to finish, prepare the buffers from the pre-lab calculations.

1. **Wash buffer**: 10 ml of final concentration:1x PBS with **20 mM** imidazole.
2. **Elution buffer**: 10 ml of final concentration:1x PBS with **250 mM** imidazole.

Begin by dispensing the appropriate volumes of 10x PBS and 1M imidazole into 15 ml conical tubes. Then add the proper amount of or nanopure water and mix by inverting the tubes or vortexing briefly.

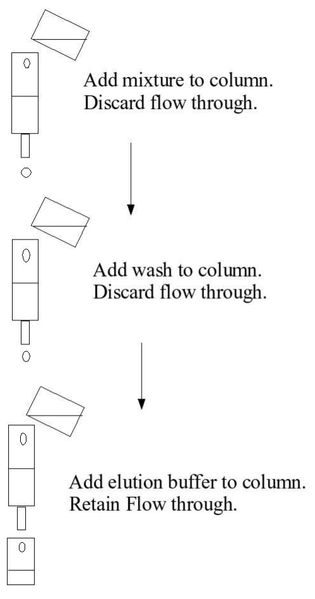
**Keep on ice**.

**Q:** Why is it important to keep your sample cold?

Syringe Filterthe lysate through a (0.22 **or** 0.45 μm) syringe filter (PES **or** SFCA) with a 5 ml syringe into a 14 ml round bottom transformation tube. These filter materials both have low protein binding and will not retain the Purple Protein but will get rid of large particulate matter. Write down which one you choose.

**Wear goggles and lab coat –filter can burst!**

**Q:** Where is your protein now?

Ni-NTA affinity purification The protein will be purified using a combination of batch and column chromatography.

First, mix the bottle of blue Ni-NTA until the mixture is homogeneous (i.e. not 2 layers). Then, to bind the protein to the resin using a batch method, add **0.5 ml** of Ni-NTA resin/buffer mix. This consists of half resin and half buffer – so that you will have 0.25 ml of resin in the end to the tube with your supernatant.

**Incubate** at room temperature for 15 minutes, mixing occasionally.

Most of the purple color should settle with the beads.

While you wait, set up the apparatus as shown in the figure – but with an ice bucket below the column. Rinse a 20 ml Bio-Rad chromatography Econo column with nanopure water and secure using a ring stand and clamp. The column should be capped with a yellow cap at the bottom so that fluid will not leak out. Keep the level of buffer above the top of the resin during all steps so that the affinity matrix does not dry out. The diagram outlines the steps involved in the process. You can visually follow the purification process and retain the drops that are purple in color.

Transfer the resin and buffer to the top of the column by pouring gently and allow the resin to settle evenly. You can rinse the conical tube with a small volume (~1 ml) of 1x PBS to dislodge anything that is stuck to the sides. After the material in the column has settled (~5 min), remove the cap and collect the clear flow through into a **10 ml** round bottom tube that is labeled with tape as “**Waste**”. Be careful to keep the level of the PBS buffer above the resin so it doesn’t dry out.

**Q:** Where is your protein now?

**Q:** What sticks to the nickel (blue stuff)?

**Q:** Why doesn’t the nickel flow through the column – isn’t Ni2+ a very small atom?

Take a **50 μl** sample of the flow through waste, dispense into a microcentrifuge tube, and label it as **sample 3** along with your **initials** and the **date**. Store this in the 4oC fridge with the other samples.

The wash step will remove proteins that are only loosely bound to the resin. Wash the Ni-NTA resin with 5 ml of 20 mM imidazole in 1x PBS by adding the buffer to the top of the column and allowing it to flow out the bottom into a different **10 ml** round bottom tube labeled “**Wash**” (again be careful with the buffer level).

**Q:** Where is your protein now?

Take a **50 μl** sample of the wash step and label it as **sample 4** along with the **date** and your **initials**. Store this in the 4oC fridge with the other samples.

The gbr22 protein can be released from the Ni-NTA resin by using a buffer with a high concentration of imidazole. Elute the bound protein by adding 5 ml of the buffer containing 250 mM imidazole to the top of the column. You can follow the gbr22 protein by monitoring the color of the solution that is dripping out the bottom. Start collecting the buffer into a clean 15 ml conical tube labeled “**Elution 1**” on ice when it begins to turn purple and collect until the solution runs clear again. Repeat by adding 5 ml of elution buffer and collecting in a 15 ml conical tube labeled “**Elution 2**”.

**Q:** Where is your protein now?

Take an Image of Elution 1 and 2 and upload to the **VDS Wikispaces page** with **captions**

(be sure you can read your tube label in the picture)

Retain these two elution samples of purified protein (label the 15 ml conical) and record the total volume collected for each.

Take a **50 μl** sample of the each Elution 1 and Elution 2 and label them as **sample 5 and sample 6** along with the date and your initials. Store this in the 4oC fridge with the **sample 1** from last week. Also store the Elution 1 and Elution 2 conical tubes in the 4oC.

**Stripping procedure**

**NOTE: do not invert your column in this step – you will lose the Ni-NTA**

The columns and affinity matrix are reusable.

To Strip the Ni-NTA, wash (let flow through) with these reagents:

10 column volumes (cv) water (cv = volume equal to amount of Ni-NTA resin that you used)  
10 cv 0.5 M NaOH (this will strip off any proteins)  
10 cv water

Cap end, Add 1 ml of 30% ethanol in Nanopure water. Place a big cap on top of the column.

You should keep resins used for different proteins separate, so label the column using tape and put your **Name**, **Date**, **VDS**, **amount** of Ni-NTA, and what **protein** you just purified, and how many times the column has been used (use **tick marks**). **Store upright in 4oC (silver mini-fridge)**

The rest of the ‘waste’ and ‘wash’ can be discarded by bleaching and pouring in drain.

Wash the 10 ml reusable round bottom tubes with Alconox soap and put in a rack to dry

**Clean your station**

**Using the Nanodrop® spectrophotometer (Thermo Scientific, Wilmington, DE)**

* This may be done independently from the rest of the lab
* One thing to do beforehand is to download the journal paper listed below and look up the maximal wavelength

UV-Vis spectroscopy can be used to estimate the concentration of your final purified protein (remember Beer’s law?). The benefit of the Nanodrop compared to the spectrophotometer that we used in lab 1 is that it does not need cuvettes and it requires only a 2 μl volume to obtain an accurate reading.

**Measuring at 280 nm**

The Nanodrop spectrophotometer is located next to the computer in bay 2. Open the software by clicking the **ND1000** icon on the desktop and select the ‘Protein A-280’ setting. To **initialize** the instrument, you will use water. Place 2 μl of nanopure water on the sample pedestal, lower the arm and click okay. Then use a tissue to remove the water, **scrub** the surface with the tissue. Then **blank** the instrument by placing 2 μl of your elution buffer on the pedestal. It is important that you blank the instrument using the buffer that your protein is in (i.e. 250 mM imidazole and 1x PBS). After taking the blank, wipe the pedestal clean. To take a reading for your protein solution, place 2 μl of your purified protein (Elution 1) on the sample pedestal. And **Measure**. The results should be recorded as the absorbance at 280 nm (A280), not as mg/ml. You will convert this reading into mg/ml based upon the information in the pre-lab that is specific for our protein. **Repeat** to get a second reading (n=2 trials). Then **Print** out the spectra of one of your readings in a small enough size to fit in your lab notebook. Also Save an Image of the spectra electronically and post it to the **VDS Wikispaces page** online. Be sure to save a copy of the electronic image of your data also - so that you will have it for your Lab Report. Select ‘Exit’ to get out of the **Protein A280 mode**

**Measuring at the MAXIMAL WAVELENGTH**

What is the wavelength in the visible region where your protein absorbs maximally?

To answer this, find the paper below and look in Table 1 (do you remember the name of our coral?):

Alieva, N. O.; Konzen, K. A.; Field, S. F.; Meleshkevitch, E. A.; Hunt, M. E.; Beltran-Ramirez, V.; Miller, D. J.; Wiedenmann, J.; Salih, A.; Matz, M. V., *Diversity and evolution of coral fluorescent proteins*. PLoS ONE **2008**, 3, (7), e2680.

Also, find the extinction coefficient at this maximal wavelength

NOTE: extinction coefficient is the same thing as molar absorptivity from Beer’s Law)

Maximal absorption wavelength \_\_\_\_\_\_\_

Extinction coefficient at Max **λ** \_\_\_\_\_\_\_

Start up the Nanodrop software again and Select the **UV/VIS mode**. **Initiatlize** with water. And **blank** with the elution buffer again. Then change the value in **λ1** to be whatever you found the MAXIMAL ABSORPTION WAVELENGTH from the instructions below.

Optionally, Set **λ**2 to be 280 nm as a comparison to your first readings above.

Take two readings (n=2) of absorbance in the visible light range

Multiply your reading by 10 (since this mode uses a 1mm path length instead of 1 cm)

Write down your results and **print** out the spectra and **save** electronic copies for **Wiki**

**Determining Concentration of protein using 280 nm WAVELENGTH**

From this protein sequence shown here, use the website below to determine the extinction coefficient at 280 nm and the molecular weight of gbr22. (Assume all Cys residues are reduced)

meffCP-gbr22 protein sequence

M S V I A K Q M T Y K V Y M S G T V N G H Y F E V E G D G K G K P Y E G E Q T V K L T V T K G G P L P F A W D I L S P L S Q Y G S I P F T K Y P E D I P D Y V K Q S F P E G Y T W E R I M N F E D G A V C T V S N D S S I Q G N C F I Y N V K I S G V N F P P N G P V M Q K K T Q G W E P N T E R L F A R D G M L I G N N F M A L K L E G G G Y Y L C E F K S T Y K A K K P V R M P G Y H Y V D R K L D V T S H N K D Y T F V E Q C E I S I A R H S L L G H H H H H H

<http://ca.expasy.org/tools/protparam.html>

Using the absorbance that you measured at 280 nm, the extinction coefficient from the website above and Beer’s law determine the concentration of your protein solution in mg/ml.

**Determining Concentration of protein using MAXIMAL WAVELENGTH**

Using this extinction coefficient at the maximal wavelength from the journal article and the absorbance at this maximal wavelength that you measured, calculate the concentration of your protein again.

Does it match what you found when you used the absorbance at 280nm?

**Q:** What actually absorbs the 280 nm wavelength?

**Determining the yield**

Based upon the volume (ml) of purified protein you collected and the concentration of this solution (mg/ml), how many mg of purified protein do you have stored in the fridge now?

This is your yield.

Show the yield if you used 280 nm wavelength \_\_\_\_\_\_\_\_\_\_\_\_\_\_

Show the yield for the Maximal Wavelength\_\_\_\_\_\_\_\_\_\_\_\_\_\_

No individual lab Report for this lab. (It will be cumulative with Protein Characterization lab)

Update your Wiki page by adding the **images** required with captions (Elution 1 tube, Elution 2 tube, Spectra).

<http://vdsstream.wikispaces.com/Protein+Labs+SP13>

**Turn in lab notebook**